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TITLE: Feasibility of Biomonitoring of Exposure to Permethrin Through Analysis of Long-Lived (Metabolite) Adducts to Proteins

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14. ABSTRACT
Biomonitoring of exposure to the insecticide permethrin is usually performed by analysis of its urinary metabolite 3-phenoxybenzoic acid (3-PBA). However, chronic low-level exposures and cumulative exposures cannot be assessed by analyzing urinary biomarkers. We are engaged in the development of a methodology to assess the cumulative internal dose of exposure to permethrin, which is based on the assumption that (reactive) glucuronide conjugates of the major permethrin metabolites 3-PBA and cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis/trans-Cl2CA) will form persistent adducts to proteins, in analogy with the glucuronide conjugates of structurally related drugs. In the second year of the project the adduct formation of the glucuronides of 3-PBA and Cl2CA was studied in plasma. A method was developed for analysis of albumin adducts of the glucuronides, which is based on pronase digestion of albumin followed by LC-tandem MS analysis of the lysine adducts. For 3-PBA glucuronide, it was attempted to quantify adduct formation by using [14C] labelled 3-PBA glucuronides, obtained by combined chemical and enzymatic synthesis. Quantitation was thwarted due to non-covalent association of the glucuronides to the proteins. It is envisaged that the obtained results will form a firm basis for development of an adduct-based methodology for biomonitoring exposure to permethrin, and that the results will give valuable toxicological information that can be used for risk assessment for the current large-scale use of permethrin.

15. SUBJECT TERMS
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SUMMARY

Biomonitoring of exposure to the insecticide permethrin is usually performed by analysis of its urinary metabolite 3-phenoxybenzoic acid (3-PBA). However, chronic low-level exposures and cumulative exposures cannot be assessed by analyzing urinary biomarkers. We are engaged in the development of a methodology to assess the cumulative internal dose of exposure to permethrin, which is based on the assumption that (reactive) glucuronide conjugates of the major permethrin metabolites 3-PBA and cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis/trans-Cl_2CA) will form persistent adducts to proteins, in analogy with the glucuronide conjugates of structurally related drugs. In the second year of the project the adduct formation of the glucuronides of 3-PBA and Cl_2CA was studied in plasma. After pronase digestion of albumin isolated from exposed human plasma, various lysine derivatives resulted with favourable mass spectrometric and chromatographic properties. A mass spectrometric method was developed for analysis of these lysine adducts, and synthetic reference standards have been prepared. In case of 3-PBA glucuronide, protein binding was provisionally quantitated by using [14C] labelled 3-PBA glucuronide, which was obtained by enzymatic synthesis. The binding studies were thwarted by non-covalent association of the glucuronides to the proteins; in the third year of the agreement more attention will be paid to this issue. It is envisaged that the obtained results can form a firm basis for development of a protein adduct-based methodology for biomonitoring of exposure to permethrin. Furthermore, the results will give valuable toxicological information that can be used for risk assessment for the large-scale use of permethrin.

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LIST OF ABBREVIATIONS

Boc	tert-butyloxycarbonyl
Cl$_2$CA	3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DIPEA	diisopropyl ethylamine
DMF	*N,N*-Dimethylformamide
EDTA	ethylenediaminetetraacetic acid
Fmoc	fluorenylmethyloxycarbonyl
HOBT	N-hydroxybenzotriazole
MeOH	methanol
NSAID	Non-Steroidal Anti-Inflammatory Drugs
3-PBA	3-phenoxybenzoic acid
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
TFA	trifluoroacetic acid
TIS	tri-isopropylsilane
TLC	thin layer chromatography
UDP	uridine diphosphate

I   INTRODUCTION

The pyrethroid permethrin is one of the most widely used insecticides. It is effective in the control of ticks, mites and lice, while having little adverse effects in humans. Its toxicity in insects, and in humans, is based on binding to sodium channels in the nervous system, leading to prolongation of the depolarizing after-potential, repetitive after-discharges and hyperexcitation (Narahashi, 2002). Recently, it has been argued that voltage-sensitive calcium channels may also be targets of pyrethroid action (Shafer and Meyer, 2004). Permethrin has been used extensively by the allied troops in the Gulf War and in operation Iraqi Freedom, *e.g.*, by impregnating it into battle dress uniforms and bed nettings. In this way permethrin can be absorbed through the skin, while oral and respiratory exposure can also occur. Probably, soldiers can be exposed to rather high doses of permethrin by migration of the compound from clothing to the skin surface (see, *e.g*., Snodgrass, 1992). Although permethrin is generally considered as a rather safe compound, a number of adverse effects have been reported. Occupationally exposed people have been reported to experience facial skin sensations. Symptoms of acute poisonings include dizziness, headache, nausea, anorexia, and fatigue. In case of heavy exposures, muscle fasciculation and altered consciousness have been reported (He et al, 1989; Aldridge, 1990). It has been shown in animal experiments that combined exposure to (high dosages) of permethrin, DEET and pyridostigmine bromide resulted in enhanced neurotoxicity, increased mortality, increased oxidative stress, and behavioral alterations (Abou-Donia et al., 1996; Abu-Qare and Abou-Donia, 2000a, 2001; Abdel-Rahman et al, 2004; for a review see Abu-Quare and Abou-Donia, 2003). On the basis thereof it was postulated that such combined exposures might have contributed to the etiology of the so-called Gulf War Illness. Therefore, careful biomonitoring of exposure to permethrin is important for the military community.

The metabolism of permethrin has been investigated in several species (Huckle et al, 1981). The major metabolites are cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis/trans Cl_2CA) and 3-phenoxybenzoic acid (3-PBA; see e.g., Tyler et al., 2000; Hardt & Angerer, 2003). The latter metabolite is formed in two phases (see Figure 1). First, esterase-mediated cleavage of the parent compound will give 3-phenoxybenzyl alcohol, while in the second phase this compound is oxidized enzymatically (Bast and Kampffmeyer, 1998; Heder et al, 2001) to 3-PBA. The 4'-hydroxy derivative of 3-PBA has been identified as the major metabolite of permethrin in the rat (Angerer and Ritter, 1997). Subsequently, phase II metabolism will give the respective conjugates, mostly glucuronides, which facilitate the excretion process.
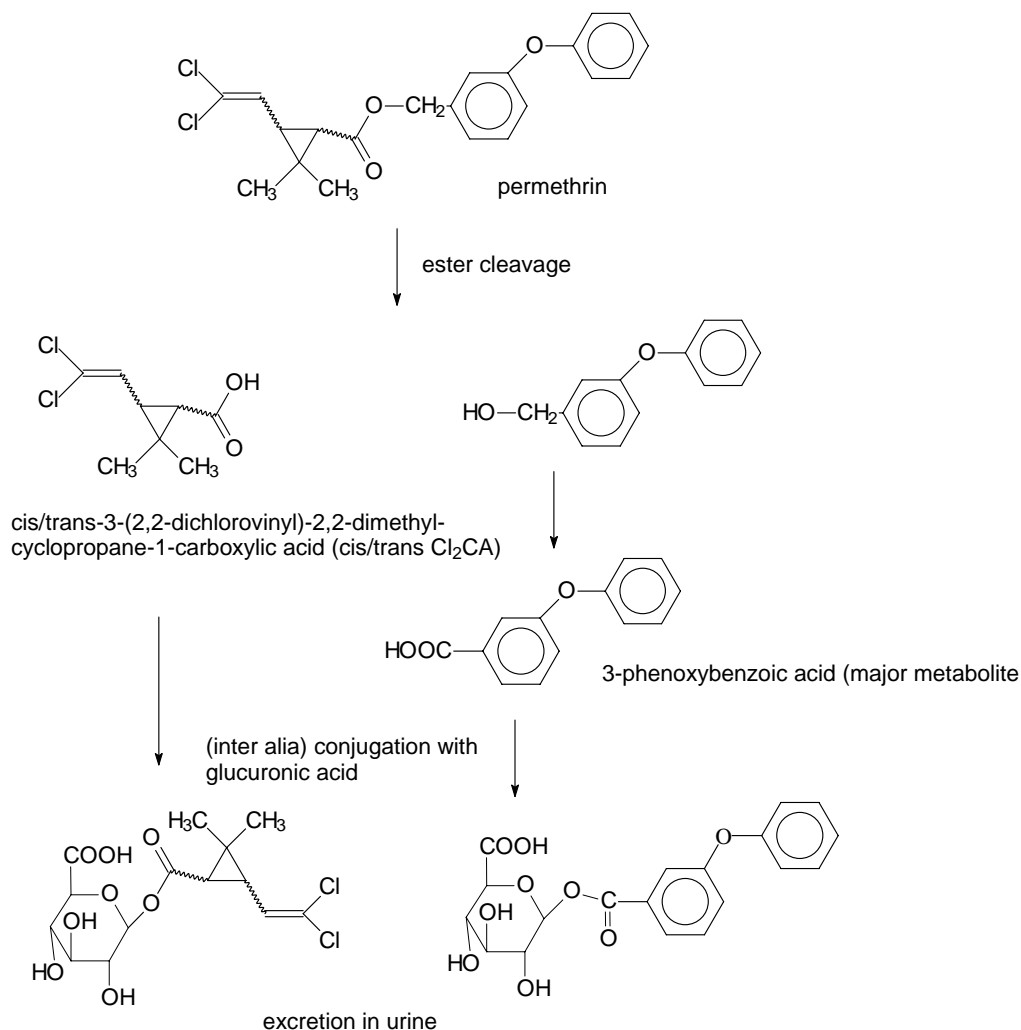
Figure 1. Metabolism of permethrin in mammals

Biomonitoring of exposure to permethrin is usually performed by analysis of its urinary metabolite 3-PBA, after acidic hydrolysis of its glucuronide (see, e.g., Hardt and Angerer, 2003; Leng et al., 2003 Abu-Qare and Abou-Donia, 2000b; Baker et al, 2004), albeit that conjugates of cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid are excreted into human urine in similar quantities as the PBA derivatives (Hardt and Angerer, 2003). Studies with a volunteer who had been exposed (orally) to the closely related pyrethroid cyfluthrin revealed that most (93%) of the urinary metabolites are excreted within 24 h (Leng et al., 1997). Consequently, urine samples for biomonitoring purposes should be taken within the first 24 h after exposure. It follows that chronic low-level exposures and cumulative doses can not be assessed by analyzing the urinary biomarkers. This observation constitutes an evident research gap.

In the current study we explore the feasibility of biomonitoring of exposure to permethrin based on the determination of long-lived protein adducts derived from metabolites of permethrin. In the first annual report we reported that the 3-PBA and Cl$_2$CA glucuronide metabolites of permethrin could be chemically synthesized. The reactivity of these

metabolites with various amino acids, peptides and albumin was also studied; various distinct adducts could be identified by LC tandem mass spectrometry.

In the present report we describe the interactions of 3-PBA- and Cl$_2$CA-glucuronide with plasma proteins and the preparation of radiochemically labelled 3-PBA-glucuronide for quantitation of protein binding. Furthermore, a method was developed for isolation and mass spectrometric analysis of adducts of the glucuronides to lysine residues in proteins.

Statement of work

The work described here is focused on the development of methods for biomonitoring exposure to permethrin, which are based on long-lived adduct with proteins. This will enable biomonitoring of chronic, low-level exposures to this compound. In order to develop such methods:

1. It will be assessed whether the potentially reactive permethrin metabolites 3-phenoxybenzoic acid glucuronide (3-PBA glucuronide) and cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid glucuronide (cis/trans-Cl$_2$CA glucuronide) can form adducts with proteins in human plasma.

2. A sensitive liquid chromatography tandem mass spectrometry procedure will be developed for the most suitable/abundant adduct with albumin, after enzymatic digestion.

3. The in vivo formation of the adduct will be further evaluated in laboratory animals. It will also be evaluated whether the adduct can be analyzed in plasma samples of US soldiers who have used permethrin extensively during deployment.

Time schedule
Year 1: 1
Year 2: 2
Year 3: 3

II   MATERIALS AND INSTRUMENTATION

II.1 Materials

The following commercially available products were used:

3-Phenoxybenzoic acid (Fluka, Zwijndrecht, The Netherlands); Cl_2CA (Specs Research Laboratory, Delft, The Netherlands). Acetonitrile (Baker Chemicals, Deventer, The Netherlands); pronase Type XIV from Streptomyces Griseus (E.C. 3.4.24.31) (Sigma Chemical Co., St. Louis, MO, U.S.A.); benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and Fmoc-amino acids (Novabiochem); trifluoroacetic acid, trypsin (Aldrich, Brussels, Belgium).

Slyde-A-Lyzer cassettes were obtained from Pierce. Centrex UF-2 10 kDa filters were obtained from Schleicher & Schuell (Dassel, Germany). Ultrafree (100 kD molecular weight cut-off; 15 ml) centrifugal ultrafilters were obtained from Millipore (Bedford, MA). Albumin affinity chromatography was carried out on HiTrap Blue HP columns (1 ml; Amersham Biosciences, Uppsala, Sweden). Desalting of albumin fractions was carried out on PD-10 columns containing Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden).

II.2 Instrumentation/devices

Liquid chromatography experiments were run on an ÁKTA explorer chromatography system (Amersham Pharmacia, Uppsala, Sweden). Columns used were a Pep RPC 5/5 column (Pharmacia, Uppsala, Sweden), a Zorbax SB C-18 column (4.6 mm x 150mm; 5 µm, Zorbax, Mac-Mod Analytical, Chadds Ford, PA, USA) and a Source 15 RPC column (Amersham Pharmacia, Uppsala, Sweden).

LC/electrospray tandem mass spectrometric analyses for obtaining structural information were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 µl injection loop mounted and a PepMap C_{18} (LC Packings) or Vydac C18 column (both 15 cm x 300 µm I.D., 3 µm particles). A gradient of eluents A (H_2O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approximately 6 µl/min through the column and into the electrospray MS interface. MS/MS product ion spectra were recorded using a cone voltage between 25 and 40 V and a collision energy between 30 and 35 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

Other mass spectrometric analyses were carried out on a TSQ Quantum Ultra mass spectrometer (Finnigan, Thermo Electron Corporations, San Jose, USA) equipped with an Acquity Sample Manager and Binary Solvent Manager (Waters, Milford, USA). For LC-MS experiments, the liquid chromatograph was connected to the mass spectrometer source via the Sample Manager equipped with a 10 µl loop and an Acquity BEH C18 column (1.7 µ particles, 1 x 100 mm; Waters, Milford, USA). The liquid chromatography system was run with a 25 minute linear gradient from 100% A to A/B 55.5/45.5 v/v (A: 0.2% formic acid in water; B: 0.2% formic acid in acetonitrile) at a flow rate of 0.09 ml/min. The TSQ Quantum Ultra mass spectrometer was operated with a spray voltage of 3 kV, a source CID of 0 V, a sheath gas pressure of 41 A.U., aux gas pressure of 2 A.U. and a capillary temperature of 350 ºC. Positive electrospray product ion spectra were recorded at an indicated collision energy of 15-20 eV, using argon as the collision gas at a pressure of 1.5 mTorr. Negative electrospray product ion SRM data was recorded at an indicated collision energy of 15-20eV.

^{1}H-NMR spectra were recorded on a Varian (Palo Alto, CA, U.S.A.) VXR 400S spectrometer operating at 400.0 MHz respectively. Chemical shifts are given in ppm relative to tetramethyl

silane. The solvent signals at 2.525 ppm (residual $Me_2SO\text{-}d_5$ in $Me_2SO\text{-}d_6$) or 7.260 ppm (residual $CHCl_3$ in $CDCl_3$) served as a reference.

Radio-HPLC analyses were performed using a series 200 HPLC pump, UV/Vis detector and Radiomatic 625 TR Flow scintillation Analyzer (all by Perkin Elmer, Shelton, CT).

Radioactivity was counted using a tri-Carb 2900 TR liquid scintillation analyzer (Packard Instrument Co., Downers Grove, Il). Liquid Scintillation Cocktail used was Hionic Fluor (PE, Shelton, CT).

III EXPERIMENTAL PROCEDURES

Incubations of plasma with 3-PBA glucuronide and Cl$_2$CA glucuronide; isolation of albumin

To 0.5 ml of human plasma was added 5 µl of a solution of 3-PBA glucuronide in various concentrations (end concentrations in plasma: 0.5 mM, 0.05 mM, 0.005 mM and 0 mM as a control). After incubation for 2h at 37ºC, 2 ml of buffer A (50 mM KH$_2$PO$_4$, pH 7.0) was added. The solutions were filtered through 45 µm acrodisc filters and albumin was isolated over a HiTrap Blue HP affinity column. This column was equilibrated with 10 ml A buffer, followed by application of the sample. The impurities were removed by flushing the column with 10 ml A buffer, followed by elution of the albumin with 3 ml B buffer (50 mM KH$_2$PO$_4$ + 1.5M KCl, pH 7.0). Between the samples the column was consecutively flushed with 5 ml B-buffer and 20 ml A-Buffer. Subsequently, the purified albumin samples were desalted over a PD-10 desalting column, which had been equilibrated with 25 ml NH$_4$HCO$_3$ solution (50mM). After applying the samples, the columns were eluted with 3 ml NH$_4$HCO$_3$. These albumin solutions were used for enzymatic digestion.

Pronase digestion

To 750 µl of the albumin solution was added 100 µl of pronase solution (10 mg/ml 50 mM NH$_4$HCO$_3$). This mixture was incubated for 2 h at 37ºC and filtered over a 10kD filter before analysis with LC-MS(/MS).

Trypsin digestion

An aliquot (0.5 ml) of the albumin solution was lyophilized and dissolved in buffer (0.3 ml; 6 M guanidine.HCl, 100 mM Tris, 1 mM EDTA, pH 8.3). To this solution was added dithiothreitol (5 mg) and the mixture was incubated for 40 min at 55ºC. Next, monoiodoacetic acid (10 mg) was added and the mixture was incubated for another 30 min at 40ºC. The solutions were transferred into a Slide-a-lyzer cassette and dialyzed overnight against 50 mM NH$_4$HCO$_3$. To the dialyzed albumin solution ($\pm$ 3 mg albumin) was added trypsin solution (30 µl; 1 µg/µl in 50 mM NH$_4$HCO$_3$). This mixture was incubated for 4 h and filtered over a 10kD filter before analysis with LC-tandem MS.

Synthesis of lysine adducts of 3-PBA and Cl$_2$CA

The synthesis was carried out on a PHB-S-TG resin (Rapp Polymere; 0.24 mmol/g resin) containing immobilized Boc-Lys(Fmoc) on a 36 µmol scale. The resin (150 mg) was swollen in DMF (3 ml) in a peptide synthesis tube for 45 min. Subsequently, the resin was treated with piperidine/DMF (8/2, v/v), 4*4 min, and washed with DMF (5x 2 ml). To the resin were added 3-PBA or Cl$_2$CA (10 eq, in 1 ml of DMF), PyBOP (10 eq, in 1 ml of DMF), HOBT (10 eq, in 1 ml of DMF) and DIPEA (20 eq). The synthesis tube was gently shaken every 15 min. After 2.5 h the resin was washed with DMF (5x 2 ml), dichloromethane (4x 0.2 ml), dichloromethane/diethyl ether (50/50, v/v; 4 x 0.2 ml) and diethylether (4x 0.2 ml), consecutively. The resin was air dried overnight. Deprotection and cleavage from the resin was carried out as follows. To the resin was added TFA/TIS (95/5, v/v; 6x 0.6 ml with 5 min interval). After the last addition, the liquid was pushed through the tube with a plunger. The cleaved lysine derivative was left for 2 h (after first addition) in the TFA/TIS solution. The solution was concentrated under a stream of nitrogen. To the concentrated compound was added diethyl ether/pentane (1/1, v/v; 10 ml), upon which a precipitate was formed. The precipitate was isolated by means of centrifugation and was washed with diethyl

ether/pentane (1/1, v/v; 10 ml). The solid material was air dried overnight, taken up in water (1 ml), and lyophilized. The compounds were analyzed by HPLC and LC-tandem MS and were considered to be pure enough (> 90%) for use as reference compounds.

Enzymatic synthesis of β-glucuronides of 3-PBA and Cl$_2$CA, followed by incubation with human plasma

First, experiments were carried out in order to demonstrate that the glucuronides of 3-PBA and Cl$_2$CA could be prepared in this way. Thus, to an Eppendorf tube containing 3-PBA (25.6 µg), a solution (250 µL) of UDP-acetylglucosamine (1.2 mM) and UDP glucuronic acid (6 mM) in Tris buffer (120 mM Tris; 1% DMSO; 6 mM MgCl$_2$; pH 7.4), a suspension of microsomes (20 mg/ml in sucrose solution; 50 µl) was added. The mixture was incubated for 4 h at 37 ˚C. Subsequently, a mixture of TFA/CH$_3$CN (4/96, v/v; 63 µl) was added in order to precipitate the proteins. The sample was centrifuged and the supernatant was analyzed by means of LC tandem MS, that demonstrated the presence of the desired glucuronide. In an analogous way, the β-glucuronide of Cl$_2$CA was prepared and its identity was confirmed by MS.

In case of exposure of human plasma to the glucuronides, the quenching step was omitted and the plasma (0.3 ml) was added directly to the glucuronidation mixture, after 4 h of incubation at 37 ºC. Albumin was isolated and digested with pronase as described above.

Synthesis of meta-bromodiphenyl ether (to be used for synthesis of [^{14}C] 3-PBA)
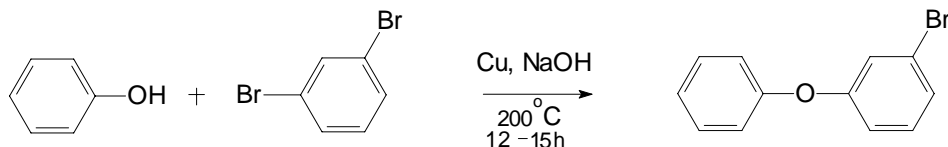
In a 50 ml 3-necked round bottom flask, equipped with a condenser, phenol (105 mmol) and NaOH (83 mmol) were stirred at 130-140 ºC (oil bath), until the NaOH had been dissolved. The reaction mixture was cooled to 100 ºC, and 8,4 mg copper powder was added. 1,3-dibromobenzene (20 ml, 0,1654 mol) was added dropwise. The temperature of the reaction mixture was increased to 200 ºC. The course of the reaction was followed by TLC. After 15 h, the reaction had gone to completion, and the reaction mixture was worked up by vacuum distillation. Further purification was performed by silica gel column chromatography (eluent: dichloromethane / hexane; gradient: 0-8% dichloromethane in hexane). Yield: 3 g (16%). Purity according to GC-MS: 97.7 %

Synthesis of 3-PBA (cold run)
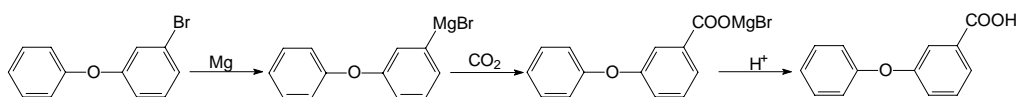
The device represented below was used for the multi-step synthesis of 3-PBA; all reagents were present in the manifold prior to the beginning of the experiment.
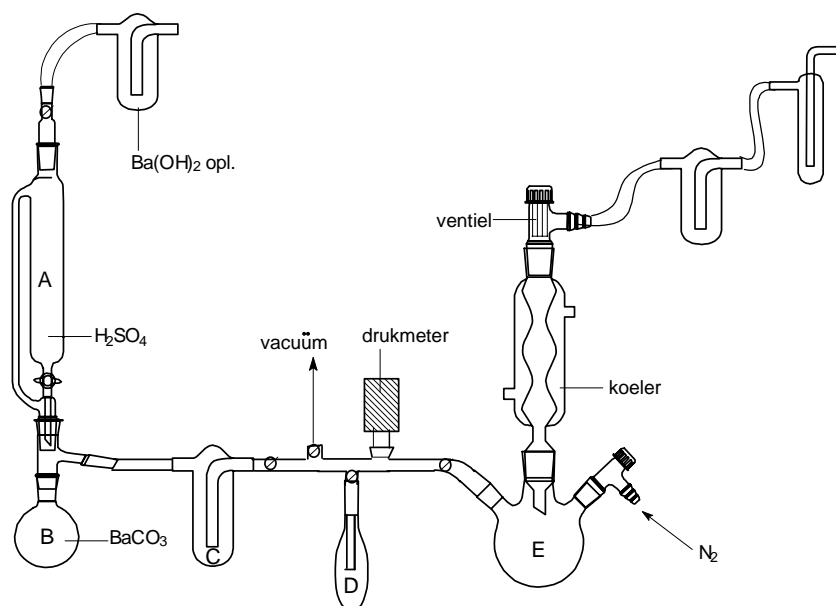

Figure 2. Manifold for preparation of [^{14}C] 3-PBA

Preparation of the Grignard reagent
To flask E, containing magnesium (52 mg, 2 mmol) and a few crystals of iodine was added a solution of 3-bromodiphenylether (750 mg, 3 mmol) in diethyl ether (3 mL). The entire system was kept under nitrogen.
The preparation of the Grignard reagent was initiated by applying heat using a heat gun. When the reaction started, the mixture was refluxed gently until all of the magnesium had disappeared. The content of the flask was frozen by using a bath of liquid nitrogen and the entire system was evacuated using a vacuum pump.

Generation of CO$_2$
The following reaction occurred:

$$BaCO_3 \text{ (s)} + H_2SO_4 \text{ (l)} \rightarrow BaSO_4 \text{ (aq)} + CO_2 \text{ (g)} + H_2O \text{ (l)}$$

Vessel A was charged with sulfuric acid (8 mL) and the left part of the manifold was brought to vacuum. By using a heat gun, the sulfuric acid was heated, until no gas development was

visible anymore. To flask B had already been added $BaCO_3$ powder (165 mg, 0.84 mmol). The left part of the manifold was evacuated again, while condenser C was cooled with solid CO_2/acetone and condenser D with liquid nitrogen. The connection between the vacuum pump and the manifold was closed and generation of CO_2 was started by dropping a few drops of concentrated sulfuric acid onto the $BaCO_3$ powder. After the first violent development of CO_2, the remaining sulfuric acid was added in a few minutes and the remaining solution was stirred and heated with a heat gun. The generated CO_2 was dried by passing through condenser C and condensed to a white solid in condenser D. Finally, the valve between condenser C and D was closed.

Synthesis of 3-PBA

The connection with the pump was closed and the valve between condenser D and the right part of the manifold was opened. Flask E was warmed up in a bath of acetone/CO_2, while condenser D was allowed to warm up to room temperature allowing the solid CO_2 to sublimate and condense in flask E. Reaction started between CO_2 and the Grignard and the reaction was stirred for another 15 min at room temperature.

Next, nitrogen was flushed through the system and water (1.5 mL) was added to the content of flask E, followed by concentrated HCl (12 M, 1.5 mL). Diethyl ether (5 mL) was added and the mixture was transferred to a separation funnel. The organic layer was extracted with water (2 x 5 mL) and evaporated to a small volume.

A solution of NaOH (0.6 g/10 mL water) was added and the solution was extracted with CH_2Cl_2 (3 x 5 mL). The water layer was acidified using concentrated HCl (3 mL) and extracted again with CH_2Cl_2 (4 x 10 mL). The combined organic layer was dried ($MgSO_4$) and concentrated. Yield 186 mg (0.87 mmol, 43 %).

Synthesis of [14C] 3-PBA

For the preparation of [14C] 3-PBA, almost the same protocol as described above was used. To flask B was added [14C]$BaCO_3$ (165 mg, 5 mCi, 0.84 mmol; sp. act 6 mCi/mmol); we used a larger excess 3-bromodiphenylether (4 mmol, 1 gram) and magnesium (3 mmol, 72 mg) than during the cold run, in order to give a maximum conversion of [14C] CO_2. After stirring the Grignard reaction mixture for 15 min, acidifying took place with HCl (4 mL, 6 M), followed by a wash step with water (3 x 3 mL). The diethyl ether layer was evaporated to dryness and dissolved in CH_2Cl_2 (10 mL) and extracted with a solution of NaOH (600 mg in water (10 mL)). The organic layer was discarded, while the alkaline water layer was extracted three more times with CH_2Cl_2 (3 x 5 mL). The water layer was acidified with HCl (12 M, 4 mL) and extracted with CH_2Cl_2 (4 x 5 mL). The organic layer was dried (MgSO4). Total yield was 0.5 mCi (10 % overall). The obtained product was purified by HPLC in order to remove a minor contamination (2-PBA; 3%). Analysis with LC/MS of the purified portion confirmed the correct mass and mass spectrum after comparison with commercial "cold" 3-PBA.

Preparation of [14C] 3-PBA-glucuronide

[14C] 3-PBA (43 microgram, 0.2 micromol, sp. act. 6 mCi/mmol) was dissolved in buffer (120 mM Tris, 1% DMSO, 6 mM $MgCl_2$, 1.2 mM UDP acetylglucosamine, 6 mM UDP glucuronic acid; 425 microliter). To this solution, a suspension of human liver microsomes (75 microliter) was added. The suspension was incubated for 4 h at 37 ºC. Reversed phase HPLC analysis showed a conversion of ca 35% to a more polar compound. The formed 3-PBA glucuronide was purified by semi-preparative HPLC. HPLC analysis of a part of the purified reaction mixture showed a single radioactive peak, having the expected retention time (comparison with synthetic reference compound).

HPLC conditions:
Buffer A: 5% CH_3CN/H_2O, 0.1 % TFA
Buffer B: 80 % CH_3CN, 0.1 % TFA

Column: Alltech C18 (250 mm * 4.6 mm, 5 micron)
HPLC system: Perkin Elmer 200 series
Detector: Perkin Elmer UV detector, 200 series
Radiomatic detector: Perkin Elmer Radiomatic flow scintillation analyzer
Gradient:
0-1 min 0% B
1-4 min 0 to 50 % B
4-19 min: 50 to 75 %B
19-20 min: 75 to 100% B
20-23 min: 100% B
23-24 min: 100 to 0 % B
24-27 min: 0 % B

MS analysis confirmed the structure of the new formed compound as [14C] 3-PBA-glucuronide (*m/z* 389 for native compound, 391 for 14C-labelled compound). Appropriate fractions were combined, lyophilized and incubated with human plasma (20 micromolar end concentration of glucuronide) for 2 h at 37 ºC.

Preparation of 3-PBA-[14C] glucuronide

To a lyophilized portion 3-PBA (43 microgram) was added a solution of [14C] UDP-glucuronic acid (250 microliter, ethanol/water 7/3 v/v, 180 mCi/mmol, 5 microCurie). The solution was evaporated to dryness in a vacuum centrifuge. Buffer (120 mM Tris, 1% DMSO, 6 mM $MgCl_2$, 1.2 mM UDP acetylglucosamine; 425 microliter) and human liver microsomes (75 microliter) were added. The suspension was incubated for 4 h at 37 ºC. HPLC analysis showed a conversion of approximately 20 % to a compound with a similar retention time as 3-PBA-glucuronide. This product was isolated using a Seppak C_{18} (classic) cartridge, because of the large difference in retention time of labeled UDP-glucuronic acid and 3-PBA glucuronide. The cartridge was conditioned with HPLC buffer B (3 mL) followed by HPLC buffer A (3 mL). The reaction mixture was diluted with water (1 mL) and applied to the Seppak cartridge, followed by 2 wash steps with buffer A (2 x 3 mL). Next, the 3-PBA-[14C]glucuronide was eluted using buffer B (2 mL). The appropriate fraction was lyophilized and used for incubations with plasma.

Incubation of [14C] labelled 3-PBA glucuronides with human plasma, sample work-up and quantitation of binding

Incubation of [14C] labelled 3-PBA glucuronides with human plasma (20 micromolar end concentration of glucuronide) was performed for 2 h at 37 ºC.
It was estimated that the protein content in plasma was 57 mg/mL.
After the incubations of purified 3-PBA-glucuronides with plasma, it was attempted to separate bound from unbound 3-PBA-glucuronides. First, molecular weight cut-off filters (0.5 mL) with a cut-off of 3 kD were used, which were washed with a solution of 20 % CH_3CN/PBS. This method was very time consuming and the use of acetonitril probably caused clogging of protein material. Use of similar filters with a cut-off of 10 kD was more successful. Small plasma samples (0.2 mL) could be washed very frequently with PBS (18 x 1 mL) at 4000g.

The PD-10 column was equilibrated with buffer (50 mM NH_4HCO_3, 25 mL). The sample was diluted to a volume of 2.5 mL with the same buffer and applied to the column. The column was eluted using 50 mM NH_4HCO_3 in 1 mL portions. From each fraction, 0.5 mL was used for liquid scintillation countings. Protein contents of PD-10 fractions were determined using a modified Lowry protocol (RC DC Protein Assay, BioRad). Protein material was usually present in fraction 3-8.

IV   RESULTS AND DISCUSSION

Adduct formation 3-PBA-glucuronide and Cl₂CA-glucuronide

Introduction

Conjugation to glucuronic acid ("glucuronidation") by UDP-glucuronosyltransferase-mediated transfer of a glucuronyl moiety of UDP-glucuronic acid to a nucleophilic site of a xenobiotic is one of the major Phase II detoxicification reactions. It renders the xenobiotic more polar which facilitates its excretion. This reaction takes place predominantly in the liver. In case of glucuronidation of a carboxylic acid, potentially reactive electrophilic acyl glucuronides result that can react with nucleophilic residues within the organism.

Two mechanisms of adduct formation by O-acyl glucuronides can be distinguished. According to the *transacylation* mechanism (Figure 3), nucleophilic sites in the proteins are acylated by the O-acyl glucuronide and consequently modified with the acyl moiety derived from the "original" metabolite. E.g., in case of the glucuronide of 3-PBA this should be a 3-phenoxybenzoyl moiety (see Figure 4 for the chemical structure of the expected adducts). According to the *glycation* mechanism, an initial internal acyl migration occurs, followed by reaction with amino groups of the protein, leading to so-called Schiff base adducts (Grubb et al., 1993; Smith et al., 1990), which may eventually undergo a (slow) Amadori rearrangement (see Figure 5). However, the latter rearrangement has not been experimentally confirmed. In case of the glucuronide of 3-PBA and Cl₂CA the expected Schiff-adduct will have the chemical structure as shown in Figure 4.
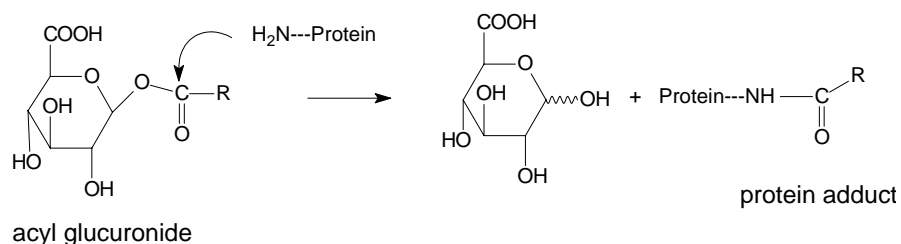

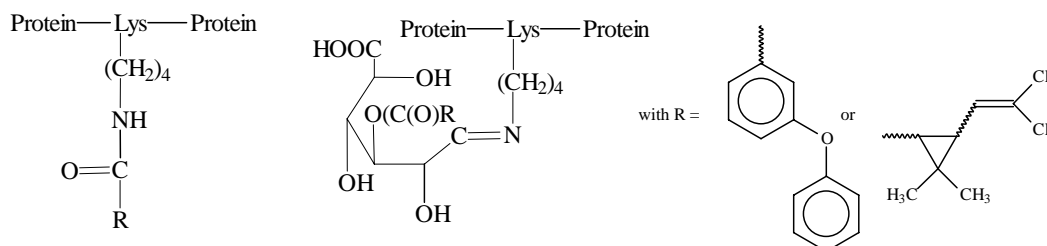**Figure 3.** Adduct formation by acyl glucuronides according to the transacylation mechanism.

Figure 4. Structure of expected lysine-adducts of permethrin-derived O-acyl glucuronides, according to the transacylation mechanism (left) and to the glycation mechanism (middle).
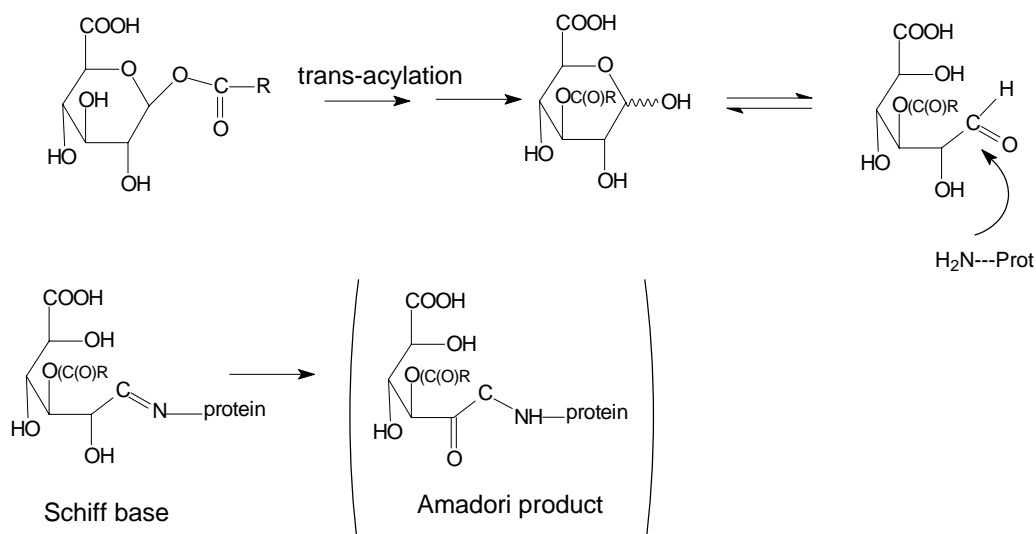
Figure 5. Adduct formation by acyl glucuronides via the glycation mechanism

In the first year of the agreement, the following results were obtained:

1. The reaction of 3-PBA glucuronide with glutathione has been shown to result in an (unstable) S-acylated adduct.
2. The reaction of 3-PBA glucuronide with model compounds has been shown to result in the formation of both adducts formed by transacylation, as well as adducts formed by the glycation mechanism.
3. For Cl_2CA glucuronide, only adducts with glutathione could be detected, derived from transacylation. No adducts could be observed after incubation of Cl_2CA glucuronide with amino-containing model compounds
4. Preliminary experiments with human plasma have been carried out. After incubation of 3-PBA glucuronide with human plasma, followed by sample work-up, two peptide adducts could be detected that were derived from albumin.

Incubations of 3-PBA and Cl_2CA β-glucuronides with human plasma, followed by enzymatic digestion

After trypsin digestion of the albumin from the 3-PBA-glucuronide exposed plasma, no ASSAKQR adducts could be detected. In the trypsin digest of plasma, incubated with 3-PBA-glucuronide, the fragment LK*ZASLQK with K* lysine modified according to the glycation mechanism was identified by LC-MS/MS. In case of exposure of plasma to Cl_2CA glucuronide, none of these peptides were found in the trypsin digest of albumin.

In addition to trypsin digestion, pronase digestion of albumin was explored. Advantage of pronase is that when modification of lysine is pronounced and quite random, the level of modified lysine in the digest will be much larger than the level of one specifically modified peptide fragment in the trypsin digest. First, the pronase digest of albumin was analyzed for

the presence of modified (acylated or glycated) Cys*-Pro-Phe, after exposure of plasma to 3-PBA glucuronide or Cl_2CA glucuronide. Probably, the adduct (a thioester or thioether) is too unstable to survive the incubation with pronase.

Next, the pronase digests were analyzed for modified lysine. In case of exposure to 3-PBA glucuronide (0.5 mM – 5 mM) peaks with MH^+ 343.1 or MH^+ 519.1 were found in all samples, corresponding to a lysine derivative resulting from transacylation or glycation, respectively. The structures of the lysine adducts were confirmed with LC-MS-MS. The adduct resulting from glycation was more intense than the peak resulting from transacylation (see Figure 6 for ion chromatogram and Figure 7 for tandem MS spectrum. Similar results were found in the pronase digests of plasma incubated with Cl_2CA glucuronide (4.6 mM). Peaks with MH^+ 337.1 and 512 (broad peak) correspond to the modified lysine derivatives. In case of the transacylation adduct, LC-MS showed two peaks for the individual isomers (see Figure 8); see Figure 9 for comparison of mass spectra of synthetic lysine derivative and of the adduct present in the digest. Interestingly, for Cl_2CA glucuronide the adduct resulting from transacylation was the more intense adduct, which is in contrast to the results obtained for 3-PBA glucuronide.
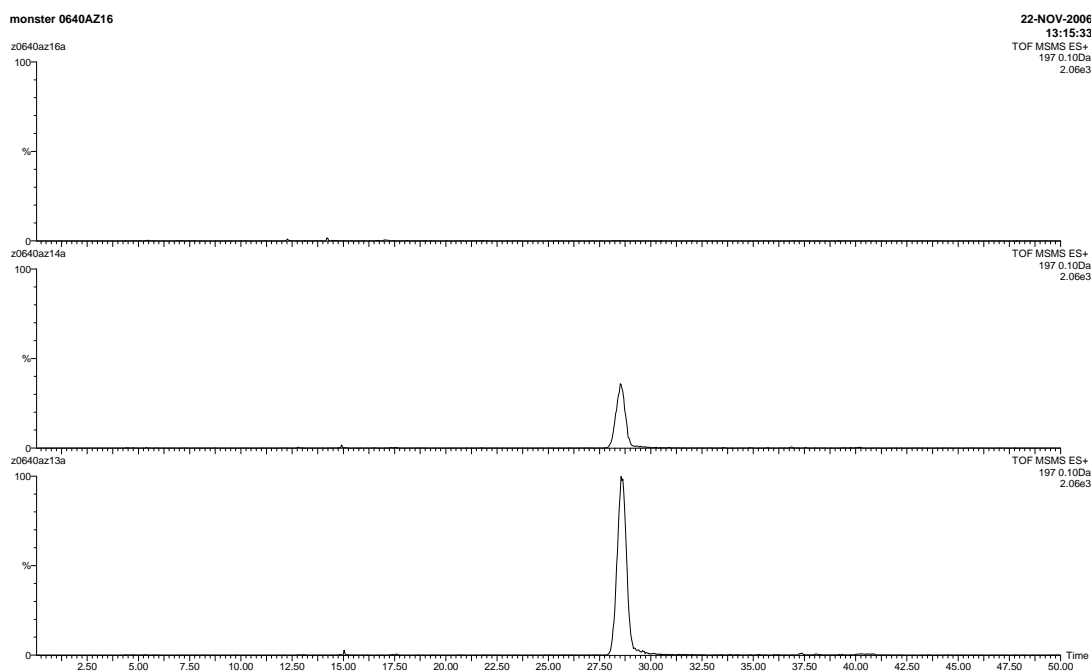

Figure 6. Ion chromatograms for 3-PBA-modified lysine (glycation adduct) in a pronase digest of albumin isolated from human plasma that was incubated with 3-PBA glucuronide. Upper trace, blank; middle trace, 1 mM exposure; 5 mM exposure.
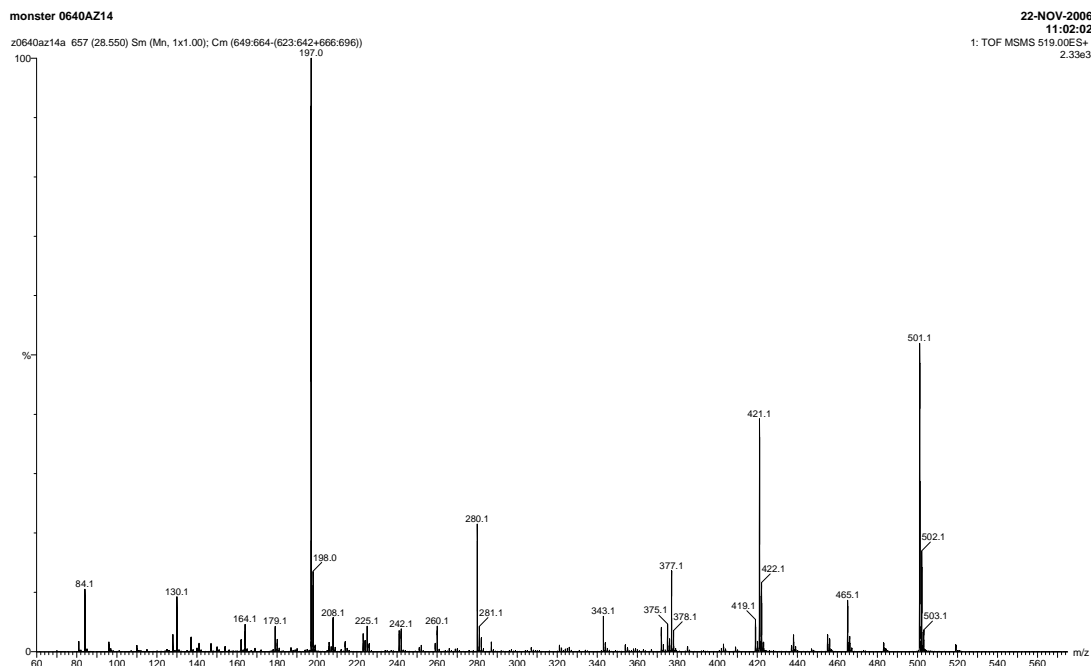
Figure 7. Tandem mass spectra of 3-PBA-lysine adduct (glycation) in a pronase digest of albumin isolated from human plasma that was incubated with 3-PBA glucuronide.
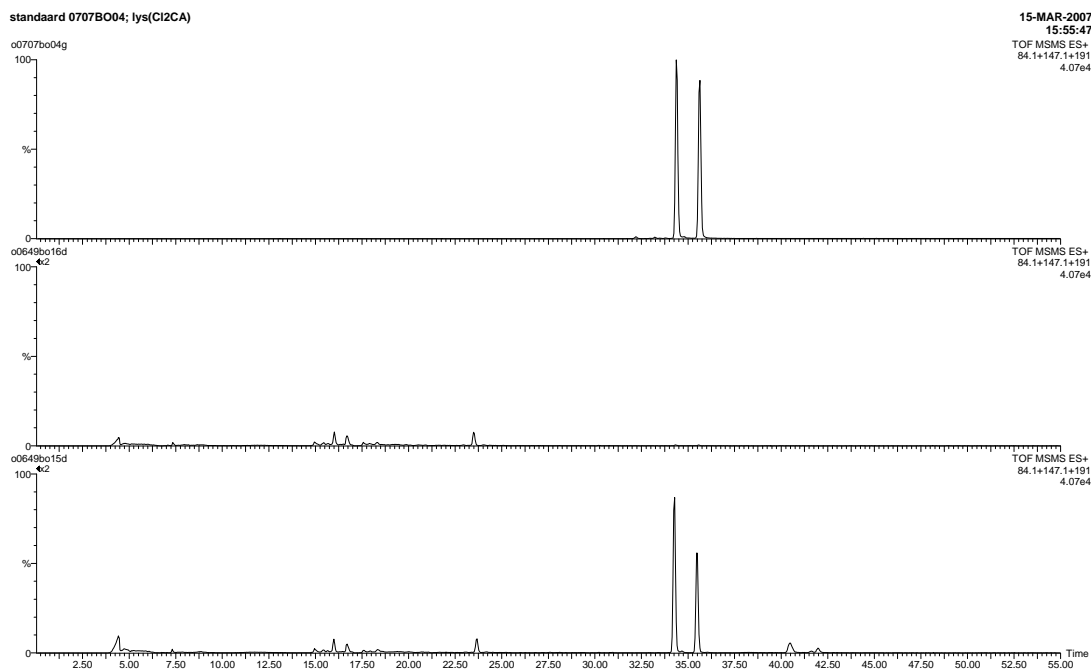

Figure 8. Ion chromatograms for Cl₂CA-modified lysine (transacylation adduct, both isomers) in a pronase digest of albumin isolated from human plasma that was incubated with synthetic Cl₂CA glucuronide. Upper trace, synthetic reference compounds; middle trace, blank plasma; exposed plasma.
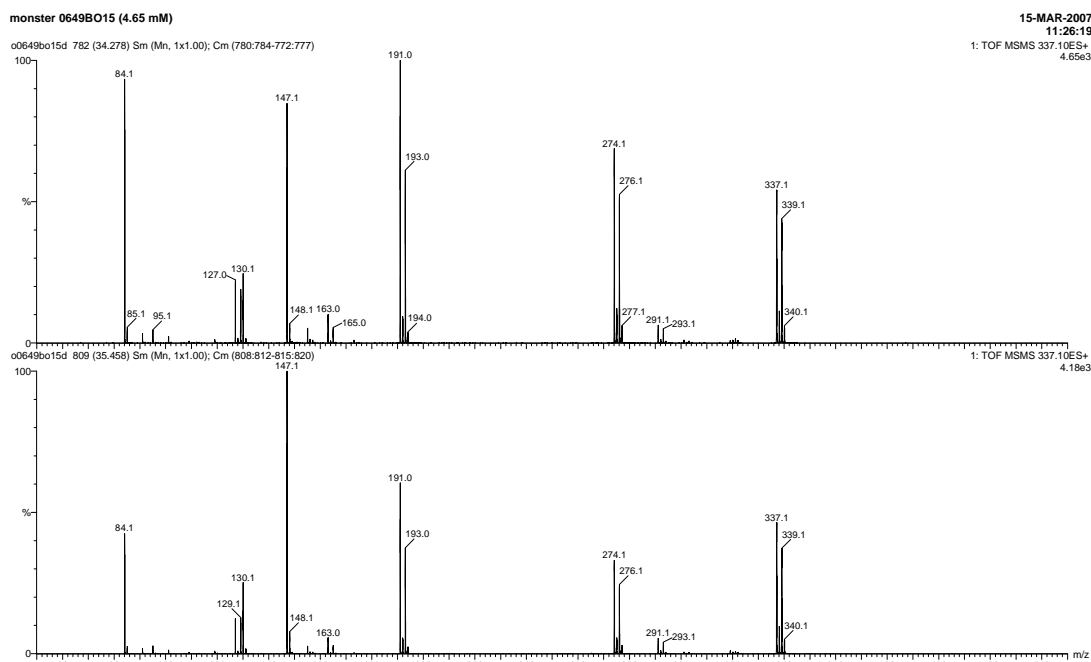
Figure 9. Tandem mass spectra of Cl$_2$CA-lysine adduct in a pronase digest of albumin isolated from human plasma that was incubated with Cl$_2$CA glucuronide. Upper trace: fast eluting isomer. Lower trace: slow eluting isomer (see Figure 8).

Care had to be taken with the results obtained with synthetic Cl$_2$CA glucuronide because a mixture of α/β-glucuronide was used. In order to ascertain that it was indeed the in vivo formed β-glucuronide was responsible for the adduct formation, we decided also to perform plasma incubations with β-glucuronide that had been prepared enzymatically, i.e., with liver microsomes and UDP-glucuronic acid. For this, the procedure published by Bolze et al (2002) was used. Plasma was exposed to the crude Cl$_2$CA-glucuronide, albumin was isolated and digested with pronase. The presence of the Cl$_2$CA-lysine adduct could be confirmed, although only one isomer of the adducts was visible, which is in contrast to the case when synthetic Cl$_2$CA-glucuronide was used (see Figure 10).
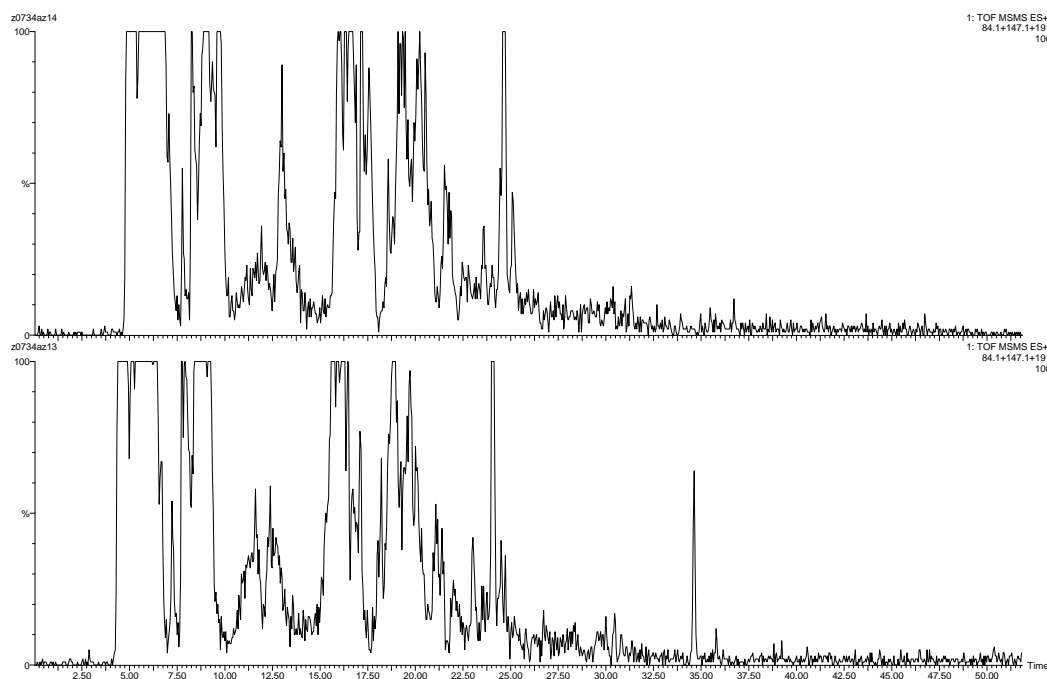
Figure 10. Ion chromatograms for Cl$_2$CA-modified lysine (transacylation adduct) in a pronase digest of albumin isolated from human plasma that was incubated with Cl$_2$CA glucuronide, that was obtained through enzymatic synthesis. Upper trace, blank plasma; lower trace, exposed plasma. Peak at 34.5 min is peak of interest.

This might be explained by the fact that during biosynthesis of the Cl$_2$CA-glucuronide the various Cl$_2$CA isomers can display different reactivity, resulting in a different ratio of β-glucuronides and probably also in a different ratio of lysine adducts. Only the lysine adduct resulting from transacylation could be observed; this adduct was also the most pronounced adduct when synthetic Cl$_2$CA glucuronide was used. The β- glucuronide of 3-PBA was also prepared enzymatically. In this case, only the glycation adduct could be observed upon exposure of plasma to the crude glucuronide (see Figure 11).

For reference purposes, the lysine adducts of 3-PBA- and Cl$_2$CA glucuronide formed by transacylation, were prepared by using a solid phase peptide synthesis protocol, starting with immobilized Boc-Lys(ε-NH-Fmoc) ; see Figure 12. The compounds displayed identical mass spectrometric properties and retention times as the lysine adducts in the pronase digests. The adducts resulting from the glycation pathway are, in this stage of the study, too complex to synthesize.
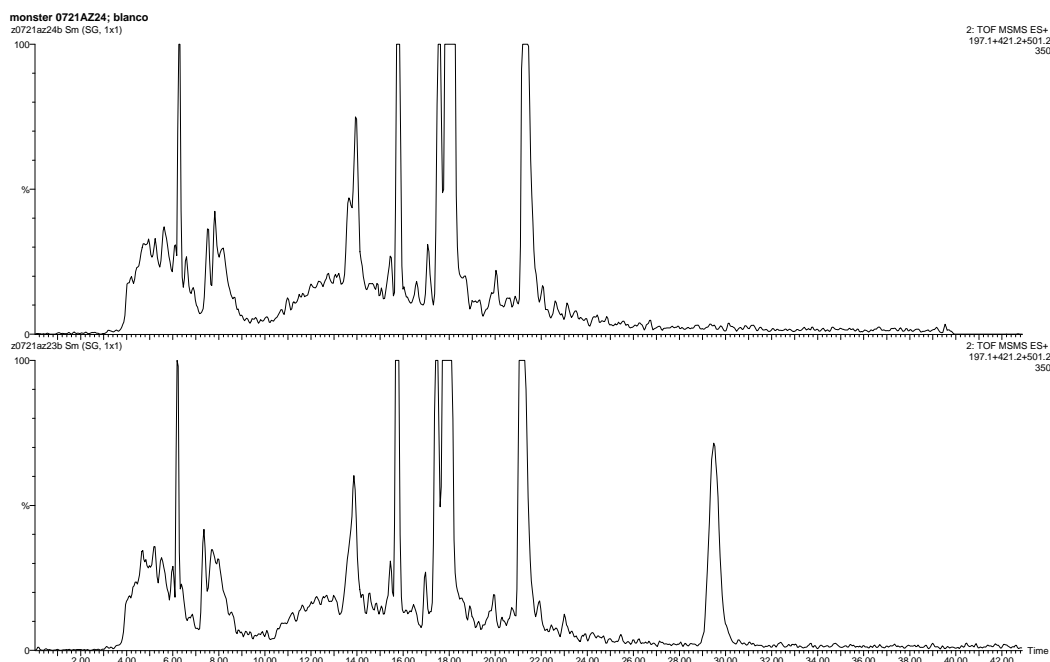
Figure 11. Ion chromatograms for 3-PBA modified lysine (glycation adduct) in a pronase digest of albumin isolated from human plasma that was incubated with 3-PBA glucuronide, that was obtained through enzymatic synthesis. Upper trace, blank plasma; lower trace, exposed plasma. Peak at 29.5 min is peak of interest.
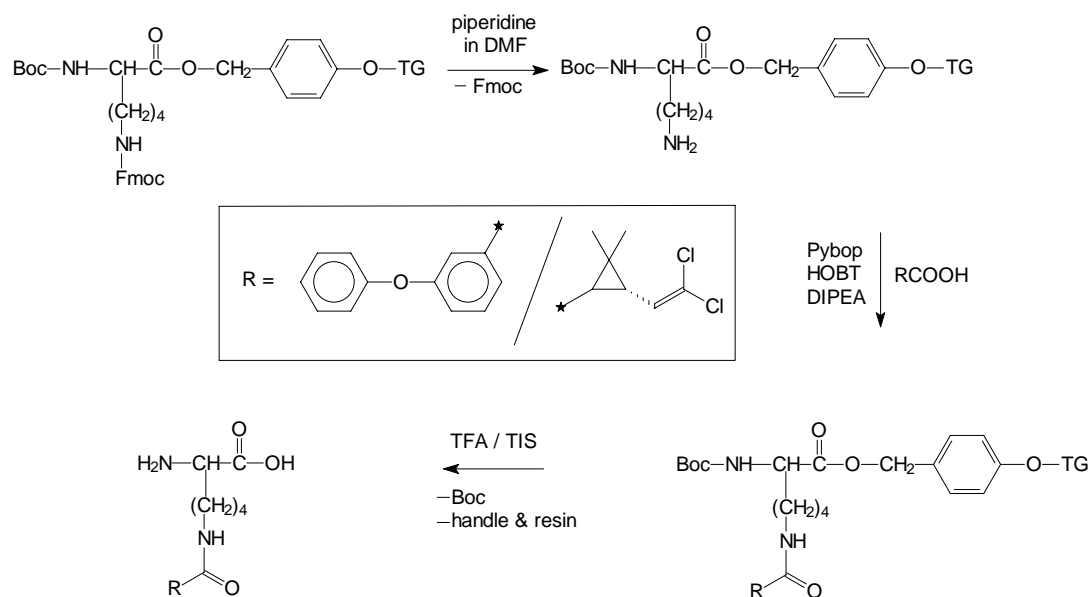

Figure 12. Synthesis scheme for lysine adducts of 3-PBA and Cl₂CA

Synthesis of [14C] 3-PBA and enzymatic synthesis of [14C] 3-PBA glucuronide

The synthesis of [14C] 3-PBA was performed by using a Grignard reaction with carbon dioxide in order to introduce the [14C] label, basically according to Elliott et al (1976). The synthetic route was first performed by using unlabelled compounds. The synthesis of the labeled material proceeded smoothly and the product displayed similar characteristics as reference 3-PBA (see Figures 13 and 14).
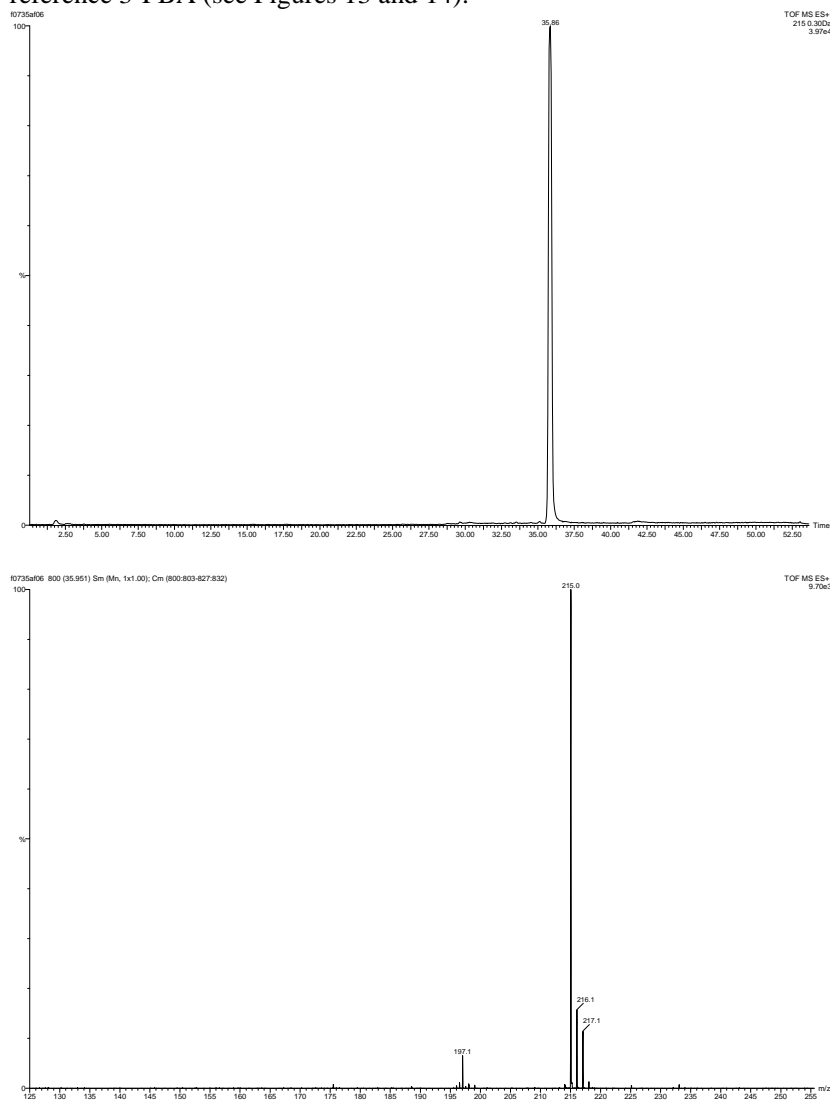

Figure 13. Mass spectrometric analysis of synthetic [14C] 3-PBA. Upper trace: total ion current. Lower trace: mass spectrum, showing the isotope ratio of labeled and unlabeled product.
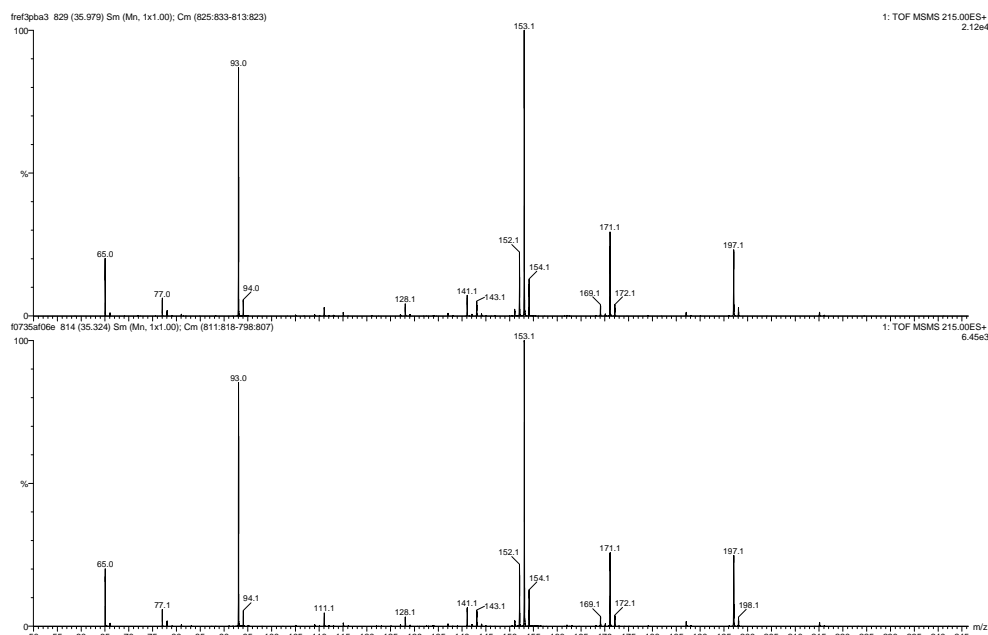
Figure 14. Comparison of commercially available reference standard 3-PBA (upper trace) and [^{14}C] 3-PBA (sp act 6 mCi/mmol), as prepared at TNO.

The enzymatic synthesis of the β-glucuronides was performed according to a literature procedure (Bolze et al., 2002). See Figure 15 for HPLC analysis (radiometric detection) of the crude incubation mixture. The glucuronide was purified by means of reversed phase HPLC. A small part of the purified material was used for thorough mass spectrometric analysis. The ion chromatogram of the isolated product is shown in Figure 16, with the respective mass spectra shown in Figure 17. As was also reported by Bolze et al (2002), the glucuronides rapidly isomerize and consequently various peaks with comparable mass spectra are observed. The mixture as such was used for incubation with plasma.
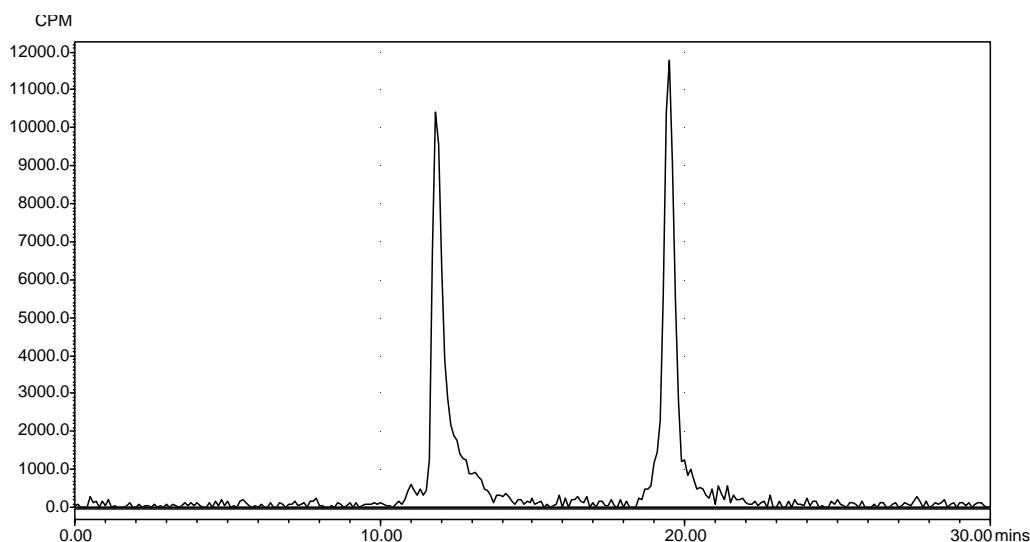
Figure 15. HPLC analysis of biosynthesis incubation mixture for synthesis of [^{14}C] 3-PBA glucuronide from [^{14}C] 3-PBA after 4 h at 37°C. The peak at 20 min represents 3-PBA, the peak at ca 12 min the glucuronide, as was confirmed by mass spectrometry.

Figure 16. Ion chromatogram of [^{14}C] 3-PBA glucuronide isomers obtained after enzymatic synthesis, after purification by reversed phase HPLC.

Figure 17. Mass spectra of isomeric [^{14}C] 3-PBA glucuronides obtained after enzymatic synthesis.

Quantitation of binding of [^{14}C] 3-PBA glucuronide in human plasma

The [^{14}C] labelled 3-PBA glucuronides were incubated with plasma for 2 h at 37 ºC. The plasma was subsequently separated into low molecular and high molecular weight material. Part of the filtrate (Figure 18) and t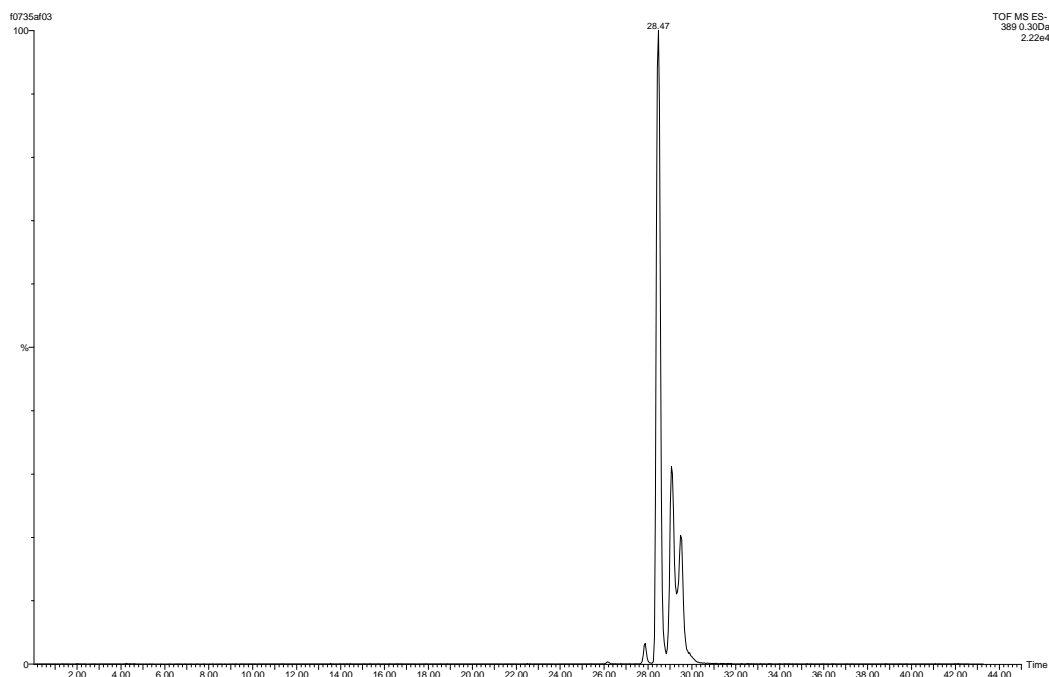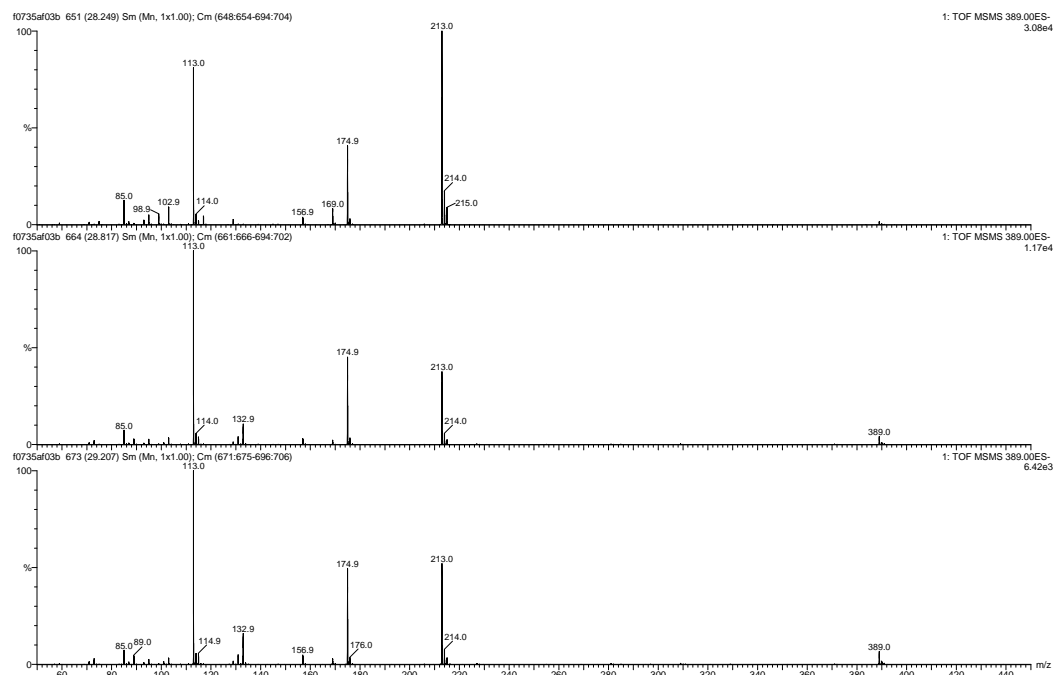he retentate (Figure 19) of the 10 kD molecular weight cut-off filter were applied to a PD-10 desalting column. The same experiment was applied to plasma exposed to [^{14}C] 3-PBA glucuronide (see Figure 20; 20 micromolar exposure level). Fractions of 1 mL were collected and analyzed for radioactivity. It was obvious that radioactivity in the retentate fractions coeluted with high molecular material, whereas the radioactivity in the filtrate fractions could be detected in low-molecular weight fractions. A plasma sample applied to a PD-10 column showed radioactivity in both high molecular as low molecular weight fractions.
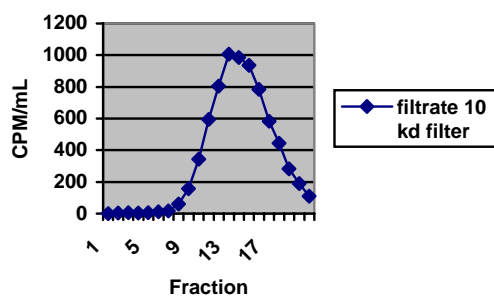

Figure 18. Radioactivity pattern after elution of a 10 kD-filtrate of a plasma sample, exposed to [^{14}C] 3-PBA-glucuronide (20 micromolar), on a PD-10 column.
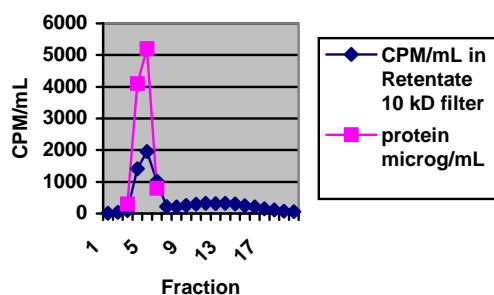
.

Figure 19. Radioactivity pattern after elution of a 10 kD retentate of a plasma sample exposed to [^{14}C] 3-PBA-glucuronide (20 micromolar), on a PD-10 column.
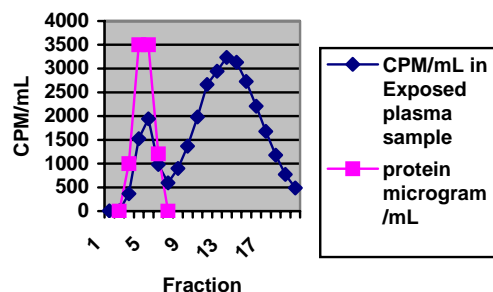
Figure 20. Radioactivity pattern (blue line) after elution of plasma (135 microliter), exposed to 20 micromolar [^{14}C] 3-PBA-glucuronide, on a PD-10 column. The pink line indicates the amount of protein.
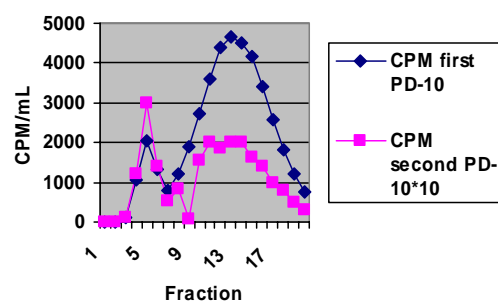

Figure 21. Radioactivity pattern after elution of a plasma sample, exposed to [^{14}C] 3-PBA-glucuronide (20 micromolar) (blue line), on a PD-10 column. The protein containing fractions (3-8) were reapplied to a PD-10 column (pink line), and after elution radioactivity was determined.

One can envisage that 3-PBA and derivatives thereof can also interact with proteins in a non-covalent way. In order to circumvent this issue, the protein-containing fractions were re-eluted over a PD-10 column (see Figure 21). Indeed, part of the radioactivity now eluted in the low-molecular weight fractions. Therefore, further quantitation of binding will be studied more careful, e.g. by extensively washing the plasma retentates on a 10 kD molecular weight cut-off filter with 6 M guanidine buffer, followed by a final PD-10 column of the retentate (in PBS), in order to get rid of all non-covalently bound material.

In conclusion, the reaction of the permethrin-derived β-glucuronides of 3-PBA and Cl$_2$CA with model peptide compounds has been shown to result in the formation of both ε-N-lysinyl adducts formed by transacylation, as well as ε-N-lysinyl adducts formed by the glycation mechanism. When incubation experiments with human plasma were carried out, adduct formation could be observed with albumin after mass spectrometric analysis of trypsin and pronase digests of albumin. Pronase digestion of albumin isolated from exposed plasma samples resulted in the formation of the various individual ε-NH$_2$-modified lysine derivatives, which can be analyzed conveniently by mass spectrometry. These adducts had favourable chromatographic and mass spectrometric properties.

In future work, the quantitation of adduct formation will be studied in more detail and animal experiments will be carried out in order to see whether adduct formation also occurs in vivo at

detectable levels. For this, the mass spectrometric method used for analysis of the lysine adduct will be further optimized. In addition, the issue of toxicological relevance of protein binding by these metabolites will be addressed in more detail. As a first step towards this, liver homogenates will be exposed to radioactively labeled glucuronides of 3-PBA, protein binding will be quantified and it will be investigated whether there are specific proteins to which these glucuronides bind.

V   KEY RESEARCH ACCOMPLISHMENTS OBTAINED IN THIS GRANT PERIOD

1. A method was developed for enzymatic synthesis of 3-PBA glucuronide and Cl_2CA glucuronide.
2. The enzymatically obtained glucuronides of 3-PBA and Cl_2CA gave also rise to adduct formation with proteins.
3. A method was developed for release and subsequent mass spectrometric analysis of adducts of 3-PBA glucuronide and Cl_2CA glucuronide to lysine residues in proteins.
4. The synthesis of lysine adducts of 3-PBA and Cl_2CA glucuronide, resulting from the transacylation mechanism, has been accomplished. These compounds can be used as reference compounds for the planned animal experiments and for eventual biomonitoring purposes.
5. The synthesis of [^{14}C] labelled 3-PBA has been accomplished.
6. The synthesis of [^{14}C] labelled 3-PBA glucuronides, either with the radioactive label in the 3-PBA moiety or in the glucuronide moiety, has been accomplished.
7. Quantitation of binding of [^{14}C] labelled 3-PBA glucuronide to plasma proteins has been undertaken. The binding studies were significantly thwarted by apparently strong non-covalent interactions of the 3-PBA derivatives with proteins. Consequently, this issue will be studied in more detail in the next grant period.

VI   REPORTED OUTCOMES (from beginning of agreement)

Publications
Biomonitoring of exposure to permethrin based on adducts to proteins
D. Noort, A. van Zuylen, A. Fidder and A.G. Hulst
NATO Human Factors & Medicine Panel Symposium 'Defense against the effects of chemical hazards. Toxicology, diagnosis, and medical countermeasures'
NATO-RTO, in press

Presentations
Generic assays for exposure biomonitoring of alkylating agents
NATO TG-009
Natick, MA, USA, October 2005
Presented by D. Noort

Persistent biomarkers of exposure
2nd International Workshop on Environmental and Health Hazards,
The Hague, The Netherlands, April 5&6, 2006
Presented by D. Noort

Persistent biomarkers of exposure of potentially neurotoxic compounds
23rd International Neurotoxicology Conference
Little Rock, USA, September 17-21, 2006
Presented by D. Noort

Biomonitoring of exposure to permethrin based on adducts to proteins
NATO Human Factors & Medicine Panel Symposium 'Defense against the effects of chemical hazards. Toxicology, diagnosis, and medical countermeasures'
October 8-10, 2007, Edinburg, UK
Presented by D. Noort

VII   CONCLUSIONS

1. The reaction of the permethrin-derived β-glucuronides of 3-PBA and Cl$_2$CA with proteins has been shown to result in the formation of both ε-N-lysinyl adducts formed by transacylation, as well as ε-N-lysinyl adducts formed by the glycation mechanism.

2. When incubation experiments with human plasma were carried out, adduct formation could be observed with albumin after mass spectrometric analysis of trypsin and pronase digests of albumin. This was the case for glucuronides prepared either by organic synthesis or by enzymatic biosynthesis.

3. Pronase digestion of albumin isolated from exposed plasma samples resulted in the formation of the various individual ε-NH$_2$-modified lysine derivatives, which can be analyzed conveniently by mass spectrometry. These adducts had favourable chromatographic and mass spectrometric properties.

4. Protein binding studies were thwarted by non-covalent interactions with (radioactively labeled) 3-PBA glucuronide or derivatives thereof; consequently, no exact data can yet be provided with regard to the degree of covalent protein binding.

5. With regard to the protein binding by the various glucuronide metabolites of permethrin, further research on the toxicological significance of this phenomenon is warranted.

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IX   BIBLIOGRAPHY OF PUBLICATIONS AND MEETING ABSTRACTS (from beginning of agreement)

Publications

Biomonitoring of exposure to permethrin based on adducts to proteins
D. Noort, A. van Zuylen, A. Fidder and A.G. Hulst
NATO Human Factors & Medicine Panel Symposium 'Defense against the effects of chemical hazards. Toxicology, diagnosis, and medical countermeasures'
NATO-RTO, in press

Meeting Abstracts

Persistent biomarkers of exposure
D. Noort
Abstract book 2nd International Workshop on Environmental and Health Hazards,
The Hague, The Netherlands, April 5&6, 2006

Persistent biomarkers of exposure of potentially neurotoxic compounds
D. Noort
Abstract book 23rd International Neurotoxicology Conference
Little Rock, USA, September 17-21, 2006

Biomonitoring of exposure to permethrin based on adducts to proteins
D. Noort
Abstract book for NATO Human Factors & Medicine Panel Symposium 'Defense against the effects of chemical hazards. Toxicology, diagnosis, and medical countermeasures'
October 8-10, 2007, Edinburg, UK

X   LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE AGREEMENT

Dr. D. Noort
Dr. B.L.M. van Baar
Mr. A. Fidder
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